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Piles of scats for piles of DNA: Deriving DNA of lizards from their faeces

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Short summary

Non-invasive genetic sampling using scats has a well established role in conservation biology; yet is rarely applied to lizard scats. We evaluated various storage and DNA extraction methods and identified a reliable method of deriving genotypes and sequences from gidgee skink scats. Results highlight the opportunity for using scat derived DNA in lizard studies, particularly for species that deposit scats in piles.

Running header A method for deriving lizard DNA from their faeces

Abstract

Species identification and distribution; individual identity and relatedness; population history, structure, and diversity and more can be derived from faecal (scat) DNA. Although there are problems, such as contamination from prey DNA, in deriving donor DNA in this way, non-invasive genetic sampling using scats has a well established role in conservation biology. Using scats from captive and wild *Egernia stokesii* (Squamata, Scincidae) we evaluated two storage and four DNA extraction methods and assessed the reliability of assessing subsequent genotypes and sequences. Reliable genotypes and sequences were obtained from frozen and dried captive lizard scat DNA extracted using a QIAamp ® DNA Stool Mini Kit and a modified Gentra ®Puregene ® method; yet success rates deteriorated for wild lizard scats. Wild *E. stokesii* eat more plants than their captive counterparts; DNA extraction may be impeded by plant inhibitors present in scats of wild lizards . Notably, reliable genotypes and sequences were obtained from wild *E. stokesii* scat DNA extracted using a Qiagen DNeasy ® Plant Mini Kit, a method designed to remove plant inhibitors. Results highlight the opportunity for using scat derived DNA in lizard studies, particularly for species that deposit scats in piles.

Introduction

Faecal pellets (scats) are a widely used source of non-invasive genetic sampling of animals (Beja-Pereira, Oliveira *et al.* 2009; Taberlet, Waits *et al.* 1999) providing information on species identification and distribution (Harris, Winnie *et al.* 2010), individual identity (Brinkman, Person *et al.* 2010), and relatedness between individuals within a population (Stenglein, Waits *et al.* 2011). At the population level, genetic data derived from scats have shed light on population history, structure, and genetic diversity (Frantz, Pope *et al.* 2003; Iyengar, Babu *et al.* 2005). Scat collection is less intrusive than most traditional methods used to extract DNA from collected tissue. It causes less stress and less disruption of normal behaviour to the study individuals (Taberlet and Luikart 1999), and may be less demanding in terms of field time, collection effort, equipment and costs (Solberg, Bellemain *et al.* 2006; Vynne, Baker *et al.* 2012). For secretive species that are hard to locate or catch, non-invasive genetic sampling using scats may be the only viable option (e.g. Alacs, Alpers *et al.* 2003). For threatened species, the use of scats can provide a means to overcome collecting permit restrictions.

However, there are specific problems in deriving donor DNA from scats. Scats usually contain some cells shed from the intestinal lining that will include DNA of the scatting individual. Scats also contain many other components including exotic DNA from food remains and gut parasites (Broquet, Menard *et al.* 2007; Marrero, Fregel *et al.* 2009; Morin, Chambers *et al.* 2001). Extraction and amplification of the DNA of the scatting individual may be inhibited by this accompanying material (Marrero, Fregel *et al.* 2009; Panasci, Ballard *et al.* 2011). Additionally, sample age and environmental conditions since the time of scat deposit can result in DNA degradation (Murphy, Kendall *et al.* 2007; Panasci, Ballard *et al.* 2011; Piggot 2004). Despite the challenges posed by the low quantity and quality of DNA, the use of scats for genetic data now has a well established place in ecological studies.

In studies of lizards, scats are widely used to derive non-genetic data on diet (Barrows 2006; Germano, Smith *et al.* 2007; Pavey, Burwell *et al.* 2010), species distribution and abundance (Turner and Medica 1982), recognition and communication (Bull, Griffith *et al.* 1999; Wilgers and Horne 2009), parasite infections (Fenner and Bull 2008; Smith, Fenner *et al.* 2009), and territoriality (Wilgers and Horne 2009). However, despite its wide application in studies of mammals, the use of scat as a DNA source in reptiles is limited to only a single published study in snakes (Jones, Cable *et al.* 2008) with none in lizards. One explanation may be that lizard scats may contain fewer cells from the scatting individual and lower DNA yields than mammal scats. In mammal scats, donor DNA is found in a mucous layer of colorectal epithelial cells that have collected on the surface of the scat as it moves through the digestive tract (Ball, Pither *et al.* 2007; Herbert, Darden *et al.* 2011; Waits and Paetkau 2005). Amplification of target DNA is more successful using the outer coating of scats than material from inside the scats (Wehausen, Ramey II *et al.* 2004), and the scat coating is regularly targeted for DNA extraction in mammal studies (Ball, Pither *et al.* 2007; Herbert, Darden *et al.* 2011; Piggot and Taylor 2003). Lizard scats appear to have a reduced mucosal coating. Despite the potential challenges, the scarcity of studies using DNA derived from lizard scats highlights an opportunity to develop this non-invasive genetic sampling method for this animal group. An important component of this process is to determine the best method for storing scat samples, and for extraction to maximise the yield of usable DNA from them.

Published studies in which DNA has been extracted from scats reveal a range of methods for scat storage. These include freezing (Nagy 2010), drying (Nsubuga, Robbins *et al.* 2004), and storage in a buffer (Frantz, Pope *et al.* 2003). Methods to extract DNA from scats also vary. For example there are off-the-shelf scat DNA extraction kits (Steinglein, Waits *et al.* 2011; Watts, Scribner *et al.* 2011), or scats can be treated using blood or tissue DNA extraction kits (Brinkman, Person *et al.* 2010; Harris, Winnie *et al.* 2010). It appears that no one method of

both storage and extraction suits all species, and Valiere, Bonenfant *et al.* (2007) and Renan, Speyer *et al.* (2012) recommended a pilot study be undertaken to identify optimal methods for a given study species. We investigated alternative methods for storing scats and deriving DNA from them for an Australian scincid lizard, *Egernia stokesii*. Our objectives were to: 1) identify optimal *E. stokesii* scat storage and DNA extraction methods; and 2) assess the reliability of DNA genotypes and sequences derived from *E. stokesii* scats using these methods. Once developed, these methods could complement traditional invasive sampling methods in this and other lizard species with the potential to increase sample size with little extra effort for several lizard species.

Materials and methods

Study species

Egernia stokesii (gidgee skink, J.E. Gray, 1845) is a large (180mm snout-vent length, Cogger (1983)), long-living, viviparous skink (Duffield and Bull 2002) widely distributed across eastern and central areas of semi-arid Australia. *Egernia stokesii* individuals live in stable family groups (Duffield and Bull 2002; Gardner, Bull *et al.* 2001a); have high levels of genetic monogamy (Gardner, Bull *et al.* 2002) and limited dispersal (Gardner, Bull *et al.* 2001b). They produce scats upon rock platforms immediately outside of the rocky crevices in which they reside, resulting in distinctive scat piles or deposits (Duffield and Bull 1998). Using olfaction, individuals can discriminate between scats from familiar group and non-group members, suggesting scat piles play an important role in social group cohesion in this species (Bull, Griffith *et al.* 1999). The use of scat derived DNA in this and similarly scat piling lizard species could provide quick access to the DNA of most group members, without the time and effort required to capture the lizards for tissue samples. Additionally, collecting scat may provide a more complete genetic sampling of social groups as some individuals may not be caught.

Scat sampling

Scats were sampled from two sources. First we used captive *E. stokesii* housed at the Flinders University of South Australia. These included individuals originally captured near Hawker (31°54′S; 138°25′E) in the southern Flinders Ranges, South Australia, during the summers of 1993-1998 and their progeny (Arida and Bull 2008; Lanham and Bull 2004; Main and Bull 1996). We randomly selected nine *E. stokesii* individuals and kept them in nine separate cages so scats could be confidently assigned to an individual. Each individual was housed in a cage (40cm high x 40cm wide x 50cm deep) in a room with a temperature of 25ºC (± 2ºC), with ceiling lights on for 12 hours a day, and heat lamps on for 6 hours a day. Scats were collected twice weekly for four weeks (total 128 scats, average 14.22 scats per lizard, SE ± 2.13). Second, scats were collected within an estimated four hours of defecation during field surveys of three *E. stokesii* populations near Hawker conducted between September 2012 and March 2013 (409 scats). Scat freshness was assessed based on colour, moisture, compaction, by the presence of a uric acid spot, and by comparison with scats of known age from the captive colony. In addition, some lizards captured during surveys defecated during handling, ensuring complete freshness of the scat samples.

In each case, scats were collected using forceps that had been cleaned in 90% ethanol between each collection, and were stored using alternative methods as described below. The diet of captive and wild *E. stokesii* differed. Captives of all ages were fed a mix of boiled eggs, fruits and vegetables, and reptile supplement, while adult wild *E. stokesii* feed largely on plant material (Duffield and Bull 1998). Based on their size, we deduced that the wild scats used in this study were from adult *E. stokesii* (Duffield and Bull 1998); this was confirmed by visual inspection which showed a largely plant derived content of wild scats.

Positive controls

To confirm that DNA derived from scats accurately represented the DNA of the scatting individual we collected blood samples from some individuals as an alternative source for DNA characterisation. Blood (up to 0.5 mL) was taken from the caudal vein of the nine isolated captive individuals and from 29 wild individuals that produced a scat while captured. Blood was stored on Whatman FTA ® Elute for later DNA extraction. We used established methods for deriving mitochondrial DNA (mtDNA) sequences and microsatellite DNA genotypes from FTA stored *E. stokesii* blood (Gardner, Bull *et al.* 2007).

Scat storage

We compared two methods of storage for the captive lizard scats. Scats were either frozen at -20°C (Frantz, Pope *et al.* 2003) (27 scats; all from the lab colony), or dried (72 scats; 54 lab colony, 18 field). Scats to be dried were sprayed with 90% ethanol and then stored on silica beads (hereafter termed dried; modified from Roeder, Archer *et al.* 2004) and kept at room temperature until DNA extraction. Samples from the field were all stored dried as this method was considered more practical for sampling in extreme conditions and away from amenities.

DNA extraction captive lizards

We trialled six DNA extraction methods using 81 scats (27 frozen, 54 dried) from captive lizards (Table 1): 1) QIAamp ® DNA Stool Mini Kit (QIAGEN ®, Catalogue 51504); 2) ISOLATE Fecal DNA Kit (Bioline ®, Catalogue BIO-52037); 3) a standard Chelex ® 100 extraction; 4) Chelex ® 100 without boiling (adopted from Casquet, Thebaud *et al.* 2011); 5) a modified Gentra ®Puregene ® (Gentra Systems) method; and 6) a direct PCR method. The first two methods were off the shelf kits specifically derived for scat samples. The next three were standard kit methods used for tissue or blood samples. The last method involved amplification without first extracting or purifying the DNA and allows for maximum

recovery of sample, but can suffer from inhibitors that are normally removed during extraction. The two kits (QIAamp and ISOLATE) were used according to manufacturer guidelines. The modified Gentra ® Puregene ® method involved immersion of the whole scat in SLP buffer (500mM Tris-HCl pH 8.0, 50mM EDTA, 10mM NaCl, (modified from Deuter, Pietsch *et al.* 1995) followed by agitation on a rotor wheel for one hour, protein precipitation with Proteinase K, DNA precipitation with ammonium acetate and isopropanol, ethanol wash, and DNA hydration in TLE buffer.

For the kit extractions, scats were selected based on recommended weight ranges where possible; *E. stokesii* scats ranged in weight from about 10 – 900mg (average 199.60mg, SE ± 16.63), therefore total weight may have been outside the recommended range (180 – 220mg QIAamp ® DNA Stool Mini Kit, up to 150mg ISOLATE Fecal DNA Kit). Where a scat was large enough, a surface scrape of the scat was used in kit extractions as this is where most of the donor individual's DNA is expected to be found. Alternatively a segment of the scat, or the entire scat was used, depending upon the protocol. In all methods, filtered pipette tips were used to minimise contamination and negative extraction controls (scat material was not added to the extraction) were used to assess contamination. Separate laboratories were used for extraction, amplification preparation and reaction. Replicate scat extractions are sometimes recommended (Taberlet, Waits *et al.* 1999) but this was not possible as a single extraction often required the whole scat to be used.

DNA amplification captive lizards

Initially, mtDNA was targeted in DNA amplification trials because cells contain more mtDNA than nuclear DNA (nuDNA), suggesting that if mtDNA could not be amplified then targeting nuDNA was likely to be futile (Taberlet, Waits *et al.* 1999). However, amplification success may be increased for smaller DNA markers (Broquet, Menard *et al.* 2007). Because only larger mtDNA genetic markers (~800bp) were currently available for *E. stokesii*, and

because scat DNA may be of low quantity and quality (Navidi, Arnheim *et al.* 1992; Taberlet, Griffin *et al.* 1996), we developed genetic markers to amplify ~200 bp of the mtDNA ND4 gene. Three primer pairs were designed in Geneious 5.6 (Biomatters Ltd 2012) based on a consensus sequence derived from 159 existing *E. stokesii* mtDNA sequences. These primer pairs were trialled in DNA derived from *E. stokesii* blood; forward primer M1544 (5'-TATGAACGCACCCATAGCCG-3') and reverse primer M1545 (5'-GCTGCTGTTAGAAGAGTGCC-3') were selected for this study.

For mtDNA only 1:5 and 1:50 dilutions were trialled. A dilution of 1:5 has previously been successful for DNA from blood in this species, but we considered that overcoming inhibitors in scat DNA may require increased dilution (Arandjelovic, Guschanski *et al.* 2009; Ball, Pither *et al.* 2007; Monteiro, Bonnemaison *et al.* 1997). DNA amplifications via polymerase chain reaction (PCR) were conducted at a total volume of 25 µl consisting of 1 x PCR Gold Buffer (Applied Biosystems), 0.20 µM of each primer, 0.80 mM dNTPs, 2 mM MgCl2, 0.5 U AmpliTaq ® Gold DNA polymerase (Applied Biosystems), 2µl of extracted DNA, and PCR grade water. The cycling conditions were nine minutes at 95 ºC, 34 cycles of 45 seconds at 94 ºC, 45 seconds at 60 ºC, one minute at 72 ºC, and a final elongation step of ten minutes at 72 ºC followed by 30 seconds at 25 ºC. To ensure that non-amplification was due to the test procedure rather than a failure of the PCR, and that positive results were not the result of contamination, one PCR positive (DNA extracted from blood and known to amplify) and two PCR negative (TLE buffer and the negative DNA extraction) controls were used in each PCR. Neat DNA from which mtDNA PCR reactions were successful was quantified using Qubit ® 2.0 Fluorometer (Life Technologies Corporation, CA); although we acknowledge that the DNA measured may have included both target and non-target DNA.

Where mtDNA amplification was successful, as determined by the presence of a band on an agarose gel, amplification trials continued using a previously developed species specific

microsatellite genetic marker (Est 1, Gardner, Cooper *et al.* 1999). For nuDNA, a QIAGEN ® Multiplex PCR Kit (QIAGEN ®, Catalogue 206143) was used. Each 10µl uniplex reaction mix contained 0.10 x QIAGEN Multiplex PCR Master Mix, 0.25 µM of each primer, 0.50 x Q-solution, 2µl of extracted DNA, and RNAse Free Water. The cycling conditions were 15 minutes at 95 ºC, 35 cycles of 30 seconds at 94 ºC, 90 seconds at 57 ºC, one minute at 72 ºC, and a final elongation step of 30 minutes at 60 ºC followed by 30 seconds at 25 ºC. Amplification success of the Est 1 locus was determined by the presence of a band on an agarose gel. A dilution of 1:50 has previously been successful for nuDNA from blood in this species. Because increased dilutions may be required to reduce the effect of inhibitors, if amplification was not successful for 1:50 dilutions, a range of DNA dilutions (neat, 1:5, 1:10, 1:100, 1:500, 1:1000) were then trialled. If Est 1 failed to amplify for any dilution, the extraction method was deemed unsuccessful for nuDNA.

DNA extraction and amplification in scats from wild lizards

Although both the QIAamp ® DNA Stool Mini Kit and modified Gentra ®Puregene ® method were successful in preliminary trials using captive lizard scats (see results), the modified Gentra ®Puregene ® method had a lower per sample cost, therefore we chose that method for validation using six wild scats; positive and negative controls were used as described for captive scats above. None of the six wild scat DNA extractions amplified for mtDNA (results not shown). We considered diet differences between captive and wild *E. stokesii* may explain differences in amplification success rates. Earlier studies have suggested that diet derived inhibitors in scats may reduce both DNA extraction yields and amplification success (Herbert, Darden *et al.* 2011; Kohn and Wayne 1997; Monroe, Grier *et al.* 2013; Panasci, Ballard *et al.* 2011). This could be particularly relevant for omnivorous or herbivorous lizards due to the presence of polysaccharides and polyphenols found in plants (Marrero, Fregel *et al.* 2009; Panasci, Ballard *et al.* 2011).

To investigate the possible presence of PCR inhibitors, DNA was extracted (using the modified Gentra ®Puregene ® method) from a further six wild scats. To test for PCR inhibition, one of the wild scat extractions was replicated in the PCR, once with only DNA extracted from the scat, and once with the scat DNA plus 2µl of a positive control. We could infer inhibitors were preventing amplification if the positive control on its own amplified but not in the reaction with scat DNA. In an effort to reduce the impact of potential inhibitors, a subset of extracted DNA from each of the six wild scats was purified using Microcon Ultracel YM-100 filters. Purified extractions were then assessed for mtDNA amplification success using the reaction mix and conditions outlined above; one sample was replicated in this PCR, one of which was spiked with the positive control to directly assess the effect of the inhibitor clean-up process (i.e. the same sample was used as in the earlier PCR).

Given the low success rates of the modified Gentra ®Puregene ® method on wild originated scat (see results), and the additional cost and effort associated with clean-up, a Qiagen DNeasy ® Plant Mini Kit was trialled for removing inhibitors. DNA was extracted from a further six wild scats according to the manufacturer instructions except initial scat sample disruption and homogenisation was avoided. Instead, the scat was left intact and, where required due to the size of the scat, additional Buffer AP1 and RNAase A stock solution (100 mg/ml) were used to ensure scats were fully immersed prior to incubation.

Validation via genotyping and sequencing

For captive samples, where an extraction method was successful, both scat and blood samples from a subset of two lizards were sequenced for the mtDNA and genotyped for seven previously described microsatellite loci (Est 1, Est 4, Est 8, Est 13, (Gardner, Cooper *et al.* 1999); TrL 28, TrL 29, TrL 35, (Gardner, Sanchez *et al.* 2008)) in PCR reactions according to the reaction mix and conditions described above except that reactions were performed in two multiplex reactions rather than uniplex (multiplex 1: Est 1, Est 4, Est 8, Est 13; multiplex

2: TrL 28, TrL 29, TrL 35). For wild samples, scat DNA of six lizards that scatted during handling was extracted using the Qiagen DNeasy ® Plant Mini Kit, and blood DNA from the same lizards, extracted using the Whatman FTA ® Elute, were similarly genotyped and sequenced. Prior to sequencing, mtDNA PCR products were purified using multiscreen PCR filter plates (Millipore Billerica, MA) to remove unincorporated primers and dNTPs. Sequence reactions were prepared using a BigDYE Terminator Cycle Sequencing Kit v3.1 (Applied Biosystems) following manufacturer recommendations, using the same primers as those used in PCR amplification. The cycling conditions were three minutes at 96 ºC, 30 cycles of 30 seconds at 96 ºC, 15 seconds at 50 ºC, four minutes at 60 ºC, and a final elongation step of three minutes at 25 ºC followed by 30 seconds at 25 ºC. Sequence products were purified using multiscreen PCR filter plates (Millipore Billerica, MA) prior to submission of DNA to the Australian Genome Research Facility (AGRF) for capillary separation on an ABI Prism 3730*xl* 96-capillary sequencer. The resulting sequences were compared against data on GenBank, to confirm species identification, using the Basic Local Alignment Search Tool (BLAST) available at http://blast.ncbi.nlm.nih.gov/Blast.cgi. For nuDNA, we compared genotypes derived from both blood and scat samples from the same individuals. PCR products were sent to the AGRF (Adelaide node) for capillary separation on an AB3730 DNA analyser; the resulting fragments were scored using GeneMapper ® (Applied Biosystems). Although recommended (Taberlet, Waits *et al.* 1999; Valiere, Bonenfant *et al.* 2007), we did not perform replicate PCRs for mtDNA or nuDNA in initial trials; instead we used blood DNA samples from the same individuals as a positive control. At this stage we were interested in determining if a sequence and genotype could be derived from scat DNA and, if they matched those derived from blood derived DNA.

Assessment of genotyping reliability

Once we identified a method for deriving genotypes from wild *E. stokesii* scats (see results), we assessed genotyping reliability using three independent PCRs (adopted from Panasci, Ballard *et al.* 2011; Stenglein, Waits *et al.* 2011). DNA amplification and genotyping were undertaken as uniplexes (see "validation via genotyping and sequencing" above).

Results

We used 81captive *E. stokesii* scats in preliminary trials (27 frozen, 54 dried, Table 1). Both frozen and dried samples amplified for both mtDNA and nuDNA (Table 1). Of the six extraction methods trialled using captive lizard scats, the QIAamp ® DNA Stool Mini Kit, ISOLATE Fecal DNA Kit, and modified Gentra ®Puregene ® methods were successful for mtDNA (Table 1). The Chelex ® 100 and direct PCR methods failed to amplify mtDNA and therefore were not trialled for nuDNA. Both the 1:5 and 1:50 DNA dilutions were successful for mtDNA, while for nuDNA, neat DNA was the most successful (results not shown). The QIAamp ® DNA Stool Mini Kit and modified Gentra ®Puregene ® methods were further tested for reliability of sequencing and genotyping using captive *E. stokesii* scats. Of twenty captive lizard scat DNA sequences derived using the QIAamp ® DNA Stool Mini Kit (n= 17) and modified Gentra ®Puregene ® method (n = 3) assessed in BLAST, 80% (n= 16) were identified as *E. stokesii*, 15% (n=3) as *Egernia sp*, and one sequence was too short to BLAST. For nuDNA, all seven microsatellite loci were successfully derived from captive lizard scat DNA extracted using both the QIAamp ® DNA Stool Mini Kit and the modified Gentra ®Puregene ® method, and all scat derived genotypes matched those derived from blood.

Based on trials using captive scats, the modified Gentra ®Puregene ® method was initially chosen for use with six wild scat samples; mtDNA amplification was unsuccessful. In subsequent trials, using a further six wild scat samples, the PCR positive control on its own was successful yet the wild scat DNA sample spiked with the positive control failed to

amplify, suggesting the presence of inhibitors. Following application of a purification method, an additional three wild scat DNA samples amplified for mtDNA. Notably, the elute DNA of six of the 12 wild scat extractions using the modified Gentra ®Puregene ® method ranged from a light tea colour to muddy brown; whereas the elute DNA of all captive scat extractions was clear.

Six wild scat samples extracted using the Qiagen DNeasy ® Plant Mini Kit were successfully sequenced and genotyped. All six samples were identified as *E. stokesii* using BLAST. All seven microsatellite DNA loci could be scored and the resulting genotypes matched those derived from blood. The elute DNA was clear for all wild scats extracted using the Qiagen DNeasy ® Plant Mini Kit. Successful mtDNA PCR reaction quantifications are available as Supplementary Material on the Journal website. The reliability of genotypes derived from DNA extracted using the Qiagen DNeasy ® Plant Mini Kit was further assessed for seven loci in five wild *E. stokesii* scat samples. All loci amplified in all replicates for all samples, except the locus for TrL 35 which failed in all replicates for one sample. Matching heterozygotes were observed in all replicates for most samples, with three exceptions. Firstly, all replicates for Est 1 and TrL 28 in one sample showed identical homozygotes. Secondly, allelic dropout was evident for Est1 in one sample which showed two matching heterozygotes and one homozygote. Lastly, for TrL 28 in one sample, two replicates showed matching homozygotes, one a heterozygote, suggesting either allelic dropout in two replicates or a false allele in one replicate.

Discussion

We have identified a reliable method for deriving DNA sequences and genotypes from wild *E. stokesii* scat samples. Genotypes and sequences were successfully derived from DNA extracted from field collected scats using a Qiagen DNeasy ® Plant Mini Kit. The reliability of genotypes derived using this method was supported by identical results from replicate

PCRs. Adoption of this method would complement traditional capture-mark-recapture methods for estimating local abundance of *E. stokesii* and other lizard species, and for estimating genetic structure and diversity, particularly for those species that create easily sampled scat piles. Co-located scats provide greater confidence of matching of scat to lizard location, making this a potentially useful tool for assessing social structures and relatedness among social group members. In addition, this method provides an alternative, non-invasive technique for threatened or secretive lizards.

Two DNA extraction methods (QIAamp ® DNA Stool Mini Kit and modified Gentra ®Puregene ® method) were successful for captive scats although success rates decreased when applied to wild scats. On the other hand, the Qiagen DNeasy ® Plant Mini Kit successfully extracted DNA from wild scats; suggesting plant inhibitors present in scats of herbivorous lizards may often prevent amplification of DNA unless they are filtered out. Although both mtDNA and nuDNA were successfully amplified from frozen and dried captive lizard scat samples, the drying method will be more suitable when sampling in semi-arid to arid locations away from electricity supplies.

As false alleles and allelic dropout may arise in scat samples with low quality and quantity of DNA (Broquet and Petit 2004; Taberlet, Waits *et al.* 1999; Valiere, Bonenfant *et al.* 2007) error checking protocols should normally be adopted. DNA amplification replicates and assessment using a consensus approach have previously been suggested (Broquet and Petit 2004; Navidi, Arnheim *et al.* 1992; Taberlet, Waits *et al.* 1999) and an assessment of power such as probability of identity is recommended (Valiere 2002). The use of replicate PCRs to assess the reliability of genotypes derived from DNA extracted, as we have doneusing the Qiagen DNeasy ® Plant Mini Kit is recommended. For this method to be adopted for this or other reptile species, a comprehensive pilot study should be undertaken that incorporates genotyping error rates into study design. In addition, as methods are not necessarily

transferable between species (Taberlet, Waits *et al.* 1999) preliminary trials are recommended prior to use with other lizard species.

There are other potential problems when deriving lizard DNA from their scats. We found reptile scales on the surface of some captive and wild scats (Pearson, *pers. obs*.). Captive lizards were isolated so it could be assumed that in those cases the scales belonged to the lizard from which scats were collected. The same assumption cannot be made for field collected scats as lizards may eat the sloughed skin of other individuals, or even conspecific neonates (Lanham and Bull 2004), potentially contaminating the sample with other conspecific DNA. Further, the co-location of scats may result in cross contamination between scats that are in contact but from different individuals. Also DNA extraction and amplification success is likely to decline with scat age as the DNA deteriorates (Demay, Becker *et al.* 2013). Wild samples used in this study were fresh; however we recommend future studies that adopt the method identified in this work consider temporal sampling thresholds.

A further potential complication is the identification of the scats of the target species. In this study, few other lizard species were sighted during the sampling of wild *E. stokesii* and the size and location of *E. stokesii* scats in piles immediately outside occupied crevice entrances facilitated identification. However, geckos were present and gecko scats may be confused with sub-adult *E. stokesii* scats, although species identification may be verified via sequencing. In addition, the field sites used in this study consisted of rocky outcrops with sparse vegetation where scats were easily found. Scats may be harder to locate and identify in an area with denser vegetation or higher lizard diversity. Knowledge of the behaviour of the target species and an awareness of other resident and transient species would be essential in such cases. However, this study indicates that more confidence may be applied to the

identification of scat from scat piling species, making non-invasive genetic sampling particularly applicable for such species.

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Figures and tables:

Table 1. Results of captive *E. stokesii* scat trials

Showing the number of scats used in trial for each storage and DNA extraction method, and numbers (*%*) successfully amplified for mt- and nuDNA for each storage and DNA extraction method.

Extraction method	Storage method		mtDNA amplified		nuDNA amplified	
	Frozen*	Dried*	Frozen	Dried	Frozen	Dried
QIAamp ® DNA Stool Mini Kit	11	20	10 (*91*)	20 (*100*)	9 (*82*)	17 (*85*)
ISOLATE Fecal DNA Kit	4	16	3 (*75*)	11 (*69*)	1 (*25*)	4 (*25*)
Direct	3	3	0	0	n/a	n/a
Chelex with boiling	3	3	0	0	n/a	n/a
Chelex without boiling	3	3	0	0	n/a	n/a
Modified Gentra ® Puregene ® method	3	9	3 (*100*)	9 (*100*)	3 (*100*)	9 (*100*)
Total	27	54	16	40	13	30

* Frozen: -20°C, Dried: sprayed with 90% ethanol then stored on silica beads at room temperature

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